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Shieh Medium Supplemented with Tobramycin for Selective Isolation of *Flavobacterium columnare* (*Flexibacter columnaris*) from Diseased Fish

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Tobramycin was found to be less inhibitory to *Flavobacterium columnare* (formerly *Flexibacter columnaris*) than to other fish-associated bacteria. The selective capacity of Shieh medium, an isolation medium described for this species, was markedly enhanced by adding tobramycin at a concentration of 1 μg/ml.

Flavobacterium columnare has been recognized as a worldwide pathogen of freshwater fish. It was formerly called *Flexibacter columnaris*, but in 1996 it was transferred to the genus *Flavobacterium* (2). *F. columnare* is the etiological agent of columnaris disease, characterized by gill necrosis, greyish white spots on the body, skin erosion, and fin rot. This etiology can be suspected by interpretation of lesions and microscopic examination of wet mounts prepared from edges of skin or gill necroses. The lesions are characterized by the presence of long, thin rods that exhibit flexing movement and are able to form columns. To verify the diagnosis, however, isolation of *F. columnare* is required. A related organism, *F. psychrophilum*, causes a systemic infection known as bacterial cold-water disease.

Aeromonas spp., *Pseudomonas* spp., and *Shewanella putrefaciens* are often present on the skin, gills, and fins of healthy or diseased fish (5, 8) and interfere with isolation of *F. columnare* due to overgrowth. Isolation of *F. columnare* from dead or moribund fish is even more difficult due to heavy postmortem or agonal growth of environmental bacteria or bacteria belonging to the normal skin flora (8). Therefore, there is a need for a reliable selective medium. Ordal and Rucker (9) considered *Cytophaga* agar to be selective for *F. columnare*. However, Fijan (6) noted that the discrete colony formation of *F. columnare* on *Cytophaga* agar was often prevented by the growth of other bacteria. He found *F. columnare* to be resistant to polymyxin B (10 U/ml) and neomycin (5 μg/ml). When these antibiotics were added to *Cytophaga* agar, the number of colonies of *F. columnare* was increased and growth of other bacteria was suppressed. The resistance of *F. columnare* to polymyxin and neomycin was also reported by Bullock et al. (3), Bernardet and Grimont (1), and Griffin (7). Another selective and differential medium was described by Shieh (10). Shieh medium has a specific salt composition. It was thought that several inorganic substances provided optimal growth circumstances for *F. columnare* (4). Song et al. (11) assessed existing media for the cultivation of *F. columnare*. They noted that Shieh medium provided better growth of *F. columnare* than *Cytophaga* agar. Of the media evaluated, generation time was the shortest in Shieh broth.

In the present communication, the use of tobramycin as a selective supplement to Shieh medium is described. We investigated the performance of the new selective medium in comparison with the polymyxin- and neomycin-supplemented medium when challenged with a number of control strains. The ability of the tobramycin-supplemented medium to recover *F. columnare* from fish with finrot or skin lesions was also assessed.

Media. Shieh medium with tobramycin at a concentration of 1 μg/ml was compared with the same medium supplemented with polymyxin (10 U/ml) and neomycin (5 μg/ml). These media were composed as follows: peptone (Difco, Detroit, Mich.), 5 g/liter; yeast extract (Difco), 0.5 g/liter; sodium acetate, 0.01 g/liter; $BaCl_2(H_2O)_2$, 0.01 g/liter; K_2HPO_4, 0.1 g/liter; KH_2PO_4, 0.05 g/liter; $MgSO_47H_2O$, 0.3 g/liter; $CaCl_22H_2O$, 0.0067 g/liter; $FeSO_47H_2O$, 0.001 g/liter; $NaHCO_3$, 0.05 g/liter; Noble agar (Difco), 10 g/liter; distilled water (pH 7.2), 1,000 ml. After sterilization (15 min, 120°C) and cooling, membrane-filtered solutions of tobramycin or polymyxin together with neomycin were added. To ensure the moisture content of the plates, the plates were poured as needed for same-day use. Unsupplemented Shieh agar and Shieh broth (composition as described above without agar) were prepared similarly.

Growth of test organisms. Nine *F. columnare*, three *F. psychrophilum*, and five other strains were used in this study. The strains and their sources are given in Table 1. Test organisms were prepared by growth for 24 h at 30°C in Shieh broth, except for *F. psychrophilum*, which was grown at 18°C. Tenfold serial dilutions of each culture were made in phosphate-buffered saline, and 0.1 ml of each dilution was inoculated on an unmodified Shieh plate and a Shieh plate supplemented with tobramycin, as well as a Shieh plate with polymyxin and neomycin. After 24 h of incubation at 18°C for *F. psychrophilum* and at 30°C for the other bacterial strains, viable counts were made simultaneously on supplemented and unsupplemented Shieh plates.

Results of comparisons made with collection strains inoculated on unsupplemented Shieh agar and Shieh agar supplemented with tobramycin or polymyxin together with neomycin are compiled in Table 2. Of the media evaluated, the tobramycin-supplemented medium revealed the strongest selectivity. Colony counts of *F. columnare* and *F. psychrophilum* strains were not or only slightly reduced. Moreover, colony sizes were similar to those on unsupplemented medium. Colony counts of *F. columnare* and *F. psychrophilum* dropped by 10^3 or 10^4 CFU on the polymyxin- and neomycin-supplemented agar in comparison with the unsupplemented medium. *Aeromonas hydrophila* 960/600, isolated from a goldfish, was capable of growing on the tobramycin-supplemented Shieh medium. However,

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TABLE 1. Sources of strains used to evaluate growth on selective medium

Species	Strain[a]	Source, year
Flavobacterium columnare	IC 8XHD5/88	United States
Flavobacterium columnare	TG 37/87	Skin ulcer, adult black bullhead, France
Flavobacterium columnare	82-3035P	United States
Flavobacterium columnare	RP TAC-4	United States
Flavobacterium columnare	GA325V	United States
Flavobacterium columnare	JIP 39/87	?
Flavobacterium columnare	CDI 363061	Fin rot, carp, The Netherlands, 1995
Flavobacterium columnare	LVDI 39/I	France
Flavobacterium columnare	NCMB 2248^T	NCMB
Flavobacterium psychrophilum	LPAA 11524	Spleen, rainbow trout fry, France
Flavobacterium psychrophilum	JIP PO2/88	Spleen, rainbow trout fry, France
Flavobacterium psychrophilum	LVDI 5/I	Gills, carp, France
Aeromonas salmonicida	NCMB 833	Brook trout
Aeromonas hydrophila	960/600	Spleen, goldfish, Belgium
Aeromonas hydrophila	960/596	Liver, carp, Belgium
Shewanella putrefaciens	NCMB 2268^T	NCMB
Pseudomonas aeruginosa	ATCC 10145^T	ATCC
Pseudomonas fluorescens	ATCC 13525^T	ATCC

[a] ATCC, American Type Culture Collection; NCMB, National Collection of Marine Bacteria.

a colony count reduction of 10^3 was noted and colony size was markedly reduced. On the polymyxin- and neomycin-supplemented agar, *A. hydrophila* 960/600 only experienced a 10-fold colony count reduction. A difference in colony size was not noted. The type strains of *A. salmonicida*, *Pseudomonas aeruginosa*, and *P. fluorescens* did not grow at all on the medium supplemented with tobramycin. They were capable of growing on the polymyxin- and neomycin-supplemented agar, although a slight colony count reduction was present. *A. hydrophila* 960/596 and *S. putrefaciens* did not grow at all on the tobramycin-supplemented medium or on the medium supplemented with polymyxin and neomycin.

Examination of samples from fish. A total of 31 mucus samples were taken from black mollies (*Poecilia sphenops*) and platys (*Xiphophorus maculatus*) with fin rot and other skin lesions, such as greyish white spots, especially on the head, and skin erosion of the back just in front of the dorsal fin. Samples were taken with a cotton swab and cultured on Shieh agar with and without tobramycin at a concentration of 1 μg/ml. All plates were incubated for 24 h at 30°C or for 48 h when *F. columnare* colonies were not clearly visible after 24 h. Identification of the isolated *F. columnare* was made on the basis of the characteristic rhizoid shape, yellow-green color, and adherence of the colonies to the agar. Furthermore, the characteristic growth in broth was evaluated along with the morphology and the typical flexing movements of the bacteria by using a light microscope (magnification, ×100). Definite identifications were made by means of a slide agglutination test with serum prepared from *F. columnare* NCMB 2248^T. Four *F. columnare* strains were isolated from mucus of the 31 diseased fish inoculated on Shieh agar supplemented with tobramycin. Growth of contaminant organisms was heavily reduced in colony size and in number, whereas heavy growth of contaminating bacteria was seen on conventional plates without the se-

TABLE 2. Growth of *F. columnare*, *F. psychrophilum*, and other fish bacteria on Shieh medium alone and on Shieh medium supplemented with tobramycin or polymyxin and neomycin

Organism	No. of CFU on:		
	Shieh agar	Shieh agar with tobramycin[a]	Shieh agar with polymyxin-neomycin[b]
F. columnare IC8XHD5/88	2×10^8	5×10^7	5×10^4
F. columnare TG 39/87	9×10^6	5×10^6	4×10^4
F. columnare 82-3035P	9×10^7	5×10^7	5×10^2
F. columnare GA325V	6×10^7	2×10^7	6×10^4
F. columnare RP TAC-4	5×10^7	2×10^7	4×10^4
F. columnare JIP 39/87	1×10^7	2×10^6	0
F. columnare CDI 363061	7×10^7	1×10^7	0
F. columnare LVDI 39/I	8×10^7	5×10^7	5×10^2
F. columnare NCMB 2248^T	9×10^6	7×10^6	9×10^4
F. psychrophilum LPAA 11524	7×10^5	5×10^5	3×10^4
F. psychrophilum JIP P02/88	6×10^5	5×10^5	2×10^4
F. psychrophilum LVDI 5/I	4×10^6	2×10^6	3×10^3
A. salmonicida NCMB 833	7×10^6	0	1×10^3
A. hydrophila 960/600	8×10^7	6×10^4	7×10^6
A. hydrophila 960/596	4×10^7	0	0
S. putrefaciens NCMB 2268	6×10^7	0	0
P. aeruginosa ATCC 10145	2×10^7	0	7×10^7
P. fluorescens ATCC 13525	2×10^8	0	7×10^7

[a] 1 μg of tobramycin/ml of Shieh agar.
[b] 10 U of polymyxin and 5 μg of neomycin/ml of Shieh agar.

lective agent. Because of this, two of the four *F. columnare* strains isolated on tobramycin-supplemented medium could not be detected on unsupplemented Shieh agar.

Polymyxin- and neomycin-supplemented agar has frequently been used for the isolation of *F. columnare*. When compared with the new medium supplemented with tobramycin, it was found to be less selective for *F. columnare*. Moreover, colony counts of this organism were more reduced than on the tobramycin-supplemented agar. Added at a concentration of 1 μg/ml, tobramycin practically did not influence the growth of pure cultures of the *F. columnare* strains tested, whereas the growth of strains belonging to other genera was clearly inhibited. These observations and the results obtained with diseased fish indicate that tobramycin could be a useful selective supplement for isolating this species. Possibly, the same supplement can be used for selective isolation of *F. psychrophilum*, but this remains to be determined with samples from diseased fish.

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